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Cellular mechanisms in sympatho-modulation of the heart

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Cardiovascular function relies on complex servo-controlled regulation mechanisms that involve both fast-acting feedback responses and long-lasting adaptations affecting the gene expression. The adrenergic system, with its specific receptor subtypes and intracellular signalling cascades provides the major regulatory system, while the parasympathetic system plays a minor role. At the molecular level, Ca^{2+} acts as the general signal trigger for the majority of cell activities including contraction, metabolism and growth. During recent years, important new results have emerged allowing an integrated view of how the multifarious Ca^{2+}-signalling mechanisms transmit adrenergic impulses to intracellular target sites. These insights into cellular and molecular mechanisms are pivotal in improving pharmacological control of the sympathetic responses to surgical trauma and perioperative stress. They are examined in detail in this review, with particular emphasis being given to the differences in intracellular signalling between cardiomyocytes and vascular smooth muscle cells.

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Heart function, in terms of contractile force (inotropy), beating frequency (heart rate) and blood supply (vascular tone), relies on a three-tiered control system: (i) immediate and fast feedback responses of the cardiac tissue to the actual mechanical load; (ii) regulation of cardiac performance by the autonomous nervous system involving humoral primary messengers affecting the intracellular signalling systems; and (iii) long-term adaptation to altered physiological and pathological conditions produced by changes in gene expression. The first two modes of regulation are partially overlapping and primarily depend on the sympathetic nervous system. Repetitive peak responses, as they occur perioperatively, are part of the life-supporting adrenergic drive, which, however, may turn into potentially life-threatening maladaptation. Gaining control over sympathetic nervous system activity by blunting the adrenergic responses to the surgical trauma and perioperative stress is an important task in anaesthetic practice. The present review summarizes substantially new experimental results on adrenergic cellular and molecular mechanisms. Because of limitations of space, reviews will often be cited where further references to the primary literature can be found. Unless otherwise stated, the molecular characteristics pertain to the human protein species. Clinical aspects of the individual sympatho-modulatory therapies in perioperative medicine, based on these new experimental findings, will be presented in the article 'Sympatho-modulatory therapies in perioperative medicine' by Zaugg and Schaub in this issue.

Adrenergic receptor subtype-specific signalling via G-proteins

Acute and long-term regulation of myocardial function, including heart rate, systolic and diastolic function and metabolism, are primarily governed by the β_1- and β_2-adrenergic receptor (AR) signalling pathways. A functional role for the α-AR is less well established, although positive and negative inotropic effects have been attributed to specific α-AR subtypes, particularly in cardiomyopathies and heart failure.[11 24 43 54 69 72 76] The human AR superfamily consists of nine subtypes originating from different genes (with single polypeptide chains varying in length from 408 to 572 amino acid residues): α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3. All these cell surface receptors

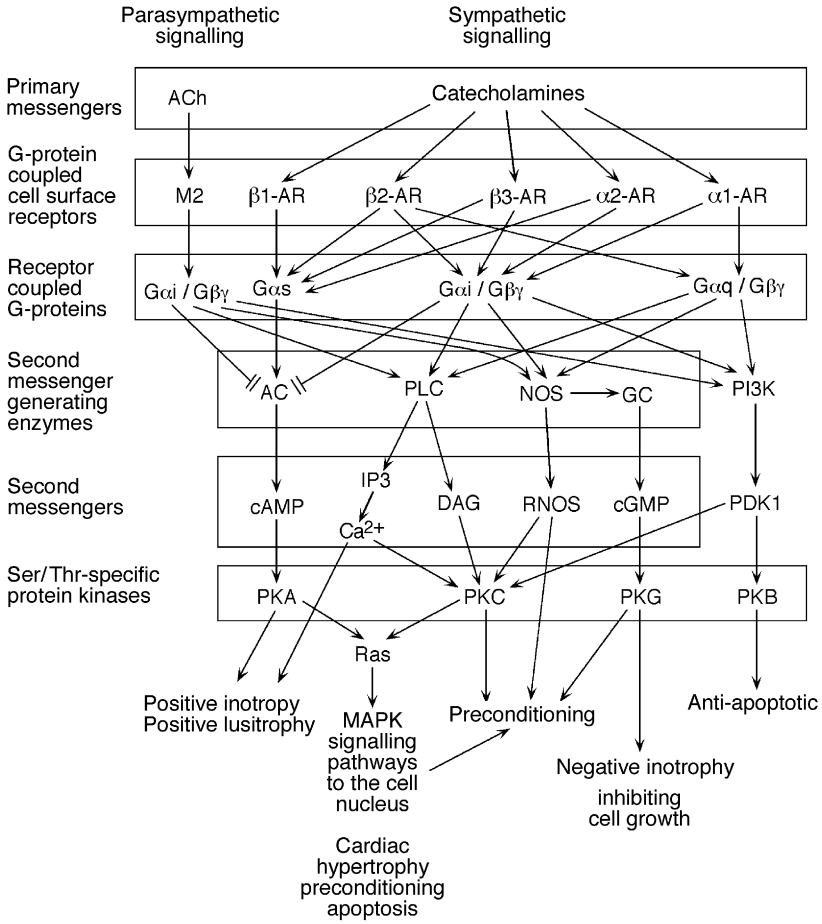

Fig 1 Sympathetic and parasympathetic signalling cascades of G-protein coupled receptors down to the level of cellular responses.[8 11 20 54 60 63 76 98 99] Note the intimate crosstalk between the various signalling pathways. Lines with blunted ends (=) indicate inhibition. AC=adenylyl cyclase; ACh=acetylcholine; AR=adrenergic receptor; cAMP=cyclic AMP; cGMP=cyclic GMP; DAG=diacylglycerol; ET1=endothelin receptor-1; GC=guanylyl cyclase; Gαi, Gαs, Gαq, Gβγ=G-protein subunits; IP3=inositol trisphosphate; M2=muscarinic acetylcholine receptor; MAPK=mitogen activated protein kinase; NOS=nitric oxide synthase; PDK1=phosphoinositide-dependent kinase-1; PI3K=phosphoinositide-3 kinase; PKA, PKB, PKC, PKG=target-specific serine–threonine protein kinases; PLC=phospholipase C; Ras=small monomeric GTPase; RNOS=reactive nitric oxide species.

contain an extracellular N-terminus, seven transmembrane α-helices (TM1–TM7) and an intracellular C-terminal region. They are all able to couple to the guanine nucleotide-binding G-proteins and are therefore called G-protein-coupled receptors (GPCRs). The extracellular N-terminal region, together with the extracellular loops between the TM2 and TM7 helices, contributes to the formation of the extracellular ligand binding pocket, whereas the amino acid sequences of the intracellular domains (loops) between the TMs, together with the proximal portion of the C-terminal region, are involved in mediating G-protein coupling. Activated G-proteins transmit signals to specific intracellular targets (Fig. 1). The downstream signalling pathways involve sequential protein phosphorylation cascades that ultimately affect targets in the cytoplasm or operate via transcriptional factors affecting gene expression. A second group of enzymes, the protein phosphatases, are responsible for dephosphorylation and termination of signalling. All intracellular signalling pathways are interconnected and form a robust network with ample redundancy. This complexity allows subtle modulation of individual cellular responses.

The GPCRs constitute the third-largest family of genes present in the human genome, and represent a central target structure for drug development. However, their therapeutic use is often limited by unwanted side-effects. Some of these derive from the binding of the drugs to related receptor subtypes.[11 72 74 76 90] In the normal human myocardium, β_1-AR is the most abundant species (70–80%), α_1 and β_2 account for over 10% each, and β_3 is low (around 1%). However, heart failure caused by either dilated or ischaemic cardiomyopathy is accompanied by selective down-regulation of β_1-AR, entailing a relative increase in both α_{1A} and β_2, which together amount to ~50% of total ARs. In the failing heart, the β_3-AR protein concentration increases by a factor of 3.

ARs couple to the heterotrimeric G-protein complex (Gα, Gβ, Gγ) on the inner side of the cell membrane.[3 12 60 76 94]

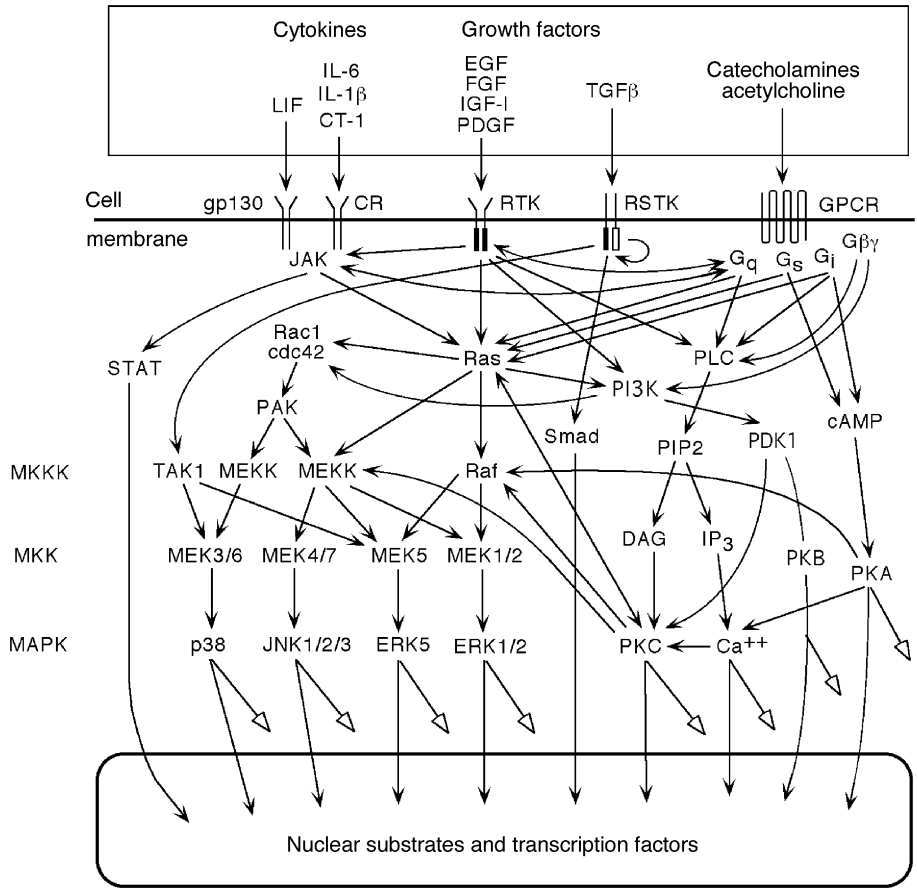

Fig 2 Interrelation of adrenergic and acetylcholine G-protein-coupled receptors (GPCR) with the global signalling pathways of the cardiomyocyte.[5 20 33 53 55 71 79 99] Open arrows indicate cytoplasmic effects. cAMP=cyclic AMP; Cdc42, Ras, Rac=small monomeric GTPases; CR=cytokine receptor; CT-1=cardiotrophin-1; DAG=diacylglycerol; EGF=epidermal growth factor; ERK=extracellular signal-regulated kinase; FGF=fibroblast growth factor; Gi, Gs, Gq, Gβγ=G-protein subunits; gp130=130 kDa leucine-rich protein; IGF-I=insulin-like growth factor-1; IL-1β, interleukin-1β; IL-6=interleukin-6; IP3=inositol trisphosphate; JAK=Janus kinase; JNK=c-Jun N-terminal kinase; LIF=leukaemia inhibitory factor; MAPK=mitogen-activated protein kinase; MEK=mitogen-activated ERK-activating kinase; MEKK=MEK kinase; MKK=MAPK kinase; MKKK=MAPKK kinase; p38=p38 MAP kinase; PAK=p21-activated kinase; PDGF=platelet-derived growth factor; PDK1=phosphoinositide-dependent kinase-1; PI3K=phosphoinositide-3 kinase; PIP2=phosphatidylinositol bisphosphate; PKA, PKB, PKC=target-specific serine–threonine protein kinases; PLC=phospholipase-C; Raf=a kinase of the MKKK family; RSTK=receptor serine–threonine kinase; RTK=receptor tyrosine kinase; Smad=TFGβ signalling protein; STAT=signal transducer and activator of transcription; TAK1=TGFβ-activated kinase-1; TGFβ=transforming growth factor-β.

Upon activation, the Gα-subunit hydrolyses guanosine triphosphate (GTP) and dissociates from the complex, leaving the βγ-subunits as undissociable heterodimer (Fig. 1). Currently there are 20 known Gα, six Gβ and eleven Gγ subunits. When activated, all three α_1-ARs interact with the pertussis toxin-insensitive Gαq component, which follows the main signalling route via phospholipase C (PLC) leading to diacylglycerol (DAG), producing activation of protein kinase C (PKC) and inositol trisphosphate (IP3) for the liberation of Ca^{2+} from the sarcoplasmic reticulum (SR). All three α_2-ARs couple to the pertussis toxin-sensitive Gαi, leading to inhibition of the integral membrane protein adenylyl cyclase (AC), activation of K^+-channels, and inhibition of the sarcolemmal L-type Ca^{2+} entry channels (DHPR). This is in contrast to the β-ARs, which are more variable in their coupling to G-proteins (Fig. 1). The β_1-AR couples almost exclusively to Gαs, inducing positive inotropy (increased contractile amplitude) and positive lusitropy (enhanced relaxation). Its canonical signalling pathways involve activation of AC and protein kinase A (PKA). β_2- and β_3-ARs are both able to signal via Gαs, Gαi or Gαq depending on the physiological or pathophysiological conditions (different states with regard to catecholamines, inflammatory cytokines and angiotensin-II). The Gαs and Gαq protein families have defined main effector pathways: the AC and the PLC pathways, respectively. The Gαi protein family is more amorphous and its signalling flows equally through both the Gαi and the Gβγ complex, affecting several different downstream signalling pathways. Gαq-dependent signalling by the heterodimeric Gβγ to the phosphoinositol-3 kinase (PI3K) pathway was recently established in cardiac hypertrophy.[20]

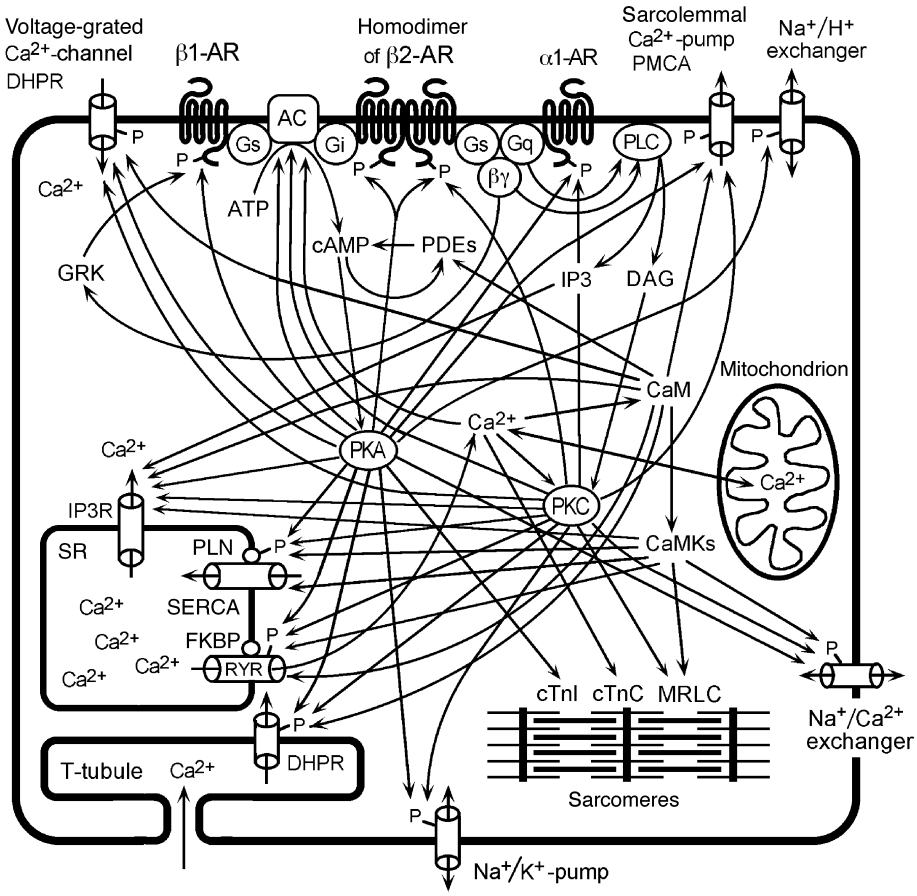

Fig 3 Interrelation of adrenergic and calcium signalling pathways affecting inotropy (contractility) and lusitropy (relaxation) in the cardiomyocyte.[7 14 21 25 42 45 82 98] AC=adenylyl cyclase; AR=adrenergic receptor; CaM=calmodulin; CaMKs=calmodulin-dependent kinases; cAMP=cyclic AMP; cTnC, cTnI=Ca^{2+}-binding and inhibitory troponin subunits respectively; DAG=diacylglycerol; DHPR=dihydropyridine receptor (L-type Ca^{2+}-channel); FKBP=FK506-binding regulatory protein; Gi, Gs, Gq, Gβγ=G-protein subunits; GRK=G-protein-coupled receptor kinase; IP3=inositol trisphosphate; IP3R=IP3 receptor (SR Ca^{2+} release channel); MRLC=myosin regulatory light chain; PDE=phosphodiesterase; PKA, PKC=target-specific serine–threonine protein kinases; PLC=phospholipase C; PLN=phospholamban; PMCA=sarcolemmal Ca^{2+} pump; RyR=ryanodine binding receptor (SR Ca^{2+} release channel); SERCA=SR Ca^{2+} pump; SR=sarcoplasmic reticulum.

AC is an integral membrane protein with 12 transmembrane helices and a molecular weight of ~130 kDa.[25 26 65] At least nine isoforms of the AC exist (AC1–AC9); AC5 is specifically expressed in cardiomyocytes, whereas AC6 occurs in heart cells other than myocytes. Additional isoforms are also expressed in the heart, but to a lesser degree. In addition to its regulation by G-proteins (stimulatory Gαs and inhibitory Gαi), PKC further stimulates and PKA inhibits the activity of AC isoforms. Furthermore, high Ca^{2+} during sustained cell activity inhibits AC5 and AC6, establishing a negative feedback loop. The fine tuning in the regulation of AC is of particular significance as its activity is rate-limiting in adrenergic signal transmission. PLC is a peripheral membrane protein at the cytoplasmic side, hydrolysing phosphatidylinositol 4,5-bisphosphate (PIP2) to DAG and IP3 (Figs 2 and 3). It has an absolute requirement for Ca^{2+} bound to the active site and comprises three types: PLCβ (150 kDa), stimulated by G-proteins; PLCγ (150 kDa), stimulated by receptor tyrosine kinases; and PLCδ (84 kDa), stimulated by transglutaminase-II.[29] All three types are expressed in cardiomyocytes.

Regulation of adrenergic receptor signalling

The β_2-AR is the most thoroughly studied with regard to signalling. Four paradigms have been delineated from studies of co-expression of wild-type and mutated β_2-AR with various combinations of G-proteins in different *in vitro* cell systems. One assumes that these regulatory mechanisms also apply to the other GPCRs in different tissues.

(i) Upon agonist activation, the β_2-AR couples to the G-protein heterotrimer, mainly with its third intracellular loop between the transmembrane helices TM5 and TM6 and part of the intracellular C-terminus immediately following the TM6. It couples preferentially to Gαs forming a stoichiometric 1:1 complex. However, the β_2-AR may instead also couple to Gαi or Gαq, activating their corresponding signalling pathways (Fig. 1). The G-protein specificity is, at least in part, determined by the type of agonist.[94] This

implies that the agonist-induced conformation of the receptor favours the type of G-protein with which it interacts. For instance, salbutamol and ephedrine display a much higher efficacy with Gαq than Gαs or Gαi. On the other hand, isoproterenol is more effective than salbutamol with Gαi. It was recently shown that the selective β_2-AR antagonist ICI-118,551 exerts a direct negative inotropic effect by acting as a β_2-AR agonist, directing it away from coupling to Gαs towards coupling to Gαi.[28] Furthermore, upon stimulation by catecholamines the β_2-AR interacts much faster with Gαs than with Gαi and Gαq. This could generate intracellular signals in a timely, ordered fashion. In general, AR signalling is terminated by phosphorylation at multiple sites in the third intracellular loop and in the C-terminal region. However, PKA-dependent phosphorylation in the third loop (serines 261 and 262) and in the proximal C-terminal region (serines 345 and 346) of the β_2-AR switches its predominant coupling from the stimulatory Gαs to the inhibitory Gαi, thereby inhibiting the AC and promoting activation of the mitogen activated protein kinase (MAPK) signalling cascade (see next section).[12 97]

(ii) Signal termination is commonly referred to as desensitization, i.e. attenuation of receptor signalling despite the continued presence of a stimulus. Desensitization may occur under physiological or pharmacological stimuli as well as under pathological conditions.[12 54 72] Homologous desensitization involves phosphorylation of two adjacent serines (355 and 356) in the C-terminal region of the β_2-AR by a family of G-protein-coupled receptor kinases (GRKs), whose activation does not require the production of second messengers. The six different GRK isoforms are tissue-specific. GRK2 and GRK5 (formerly referred to as β-ARKs; 68 and 80 kDa respectively) are expressed in cardiomyocytes and display specificity for agonist-activated receptors; non-activated receptors or antagonist-bound receptors are usually not phosphorylated by GRKs. In contrast to β_1- and β_2-AR, the β_3-AR lacks phosphorylation sites and is refractory to desensitization by GRKs or PKA. The Gβγ subunits seem to play a role in recruiting the cytoplasmic GRKs to the membrane environment of their receptor substrates and the membrane phospholipids required for kinase activation.[12 54] Heterologous desensitization occurs in the absence of agonist occupancy and is regulated by the signalling of another receptor via an intermediary second messenger. Induction of cAMP by receptor signalling leads to phosphorylation of several intracellular serines of the β_2-AR by cAMP-dependent PKA. Thus, phosphorylation by PKA turns off signalling through the receptor's normal partner Gαs and, at the same time, facilitates receptor coupling to the inhibitory Gαi.[12 21 97] Stimulation of PKC via the PLC–PIP2–DAG pathway also leads to phosphorylation of the receptor and its heterologous desensitization.

Desensitization is an acute response involving binding of arrestin to the phosphorylated C-terminus of the β_2-AR. Arrestin, together with the heterotetrameric adapter complex AP2, delivers the receptor to clathrin-coated pits for endocytosis to endosomes or to lysosomes. In the endosomes the β_2-AR is dephosphorylated by specific protein phosphatases. This resensitized receptor then recycles back to the cell membrane. However, fusion of the endosome with lysosomes leads to β_2-AR degradation. Desensitization is not always coupled to internalization but exhibits receptor type-specificity. For instance, under agonist stimulation 50–80% of the β_2-AR internalizes within a few minutes, whereas the β_1-AR does not internalize but remains at the cell surface, even in the desensitized state. The different α-ARs internalize only moderately, except for the α_{2A}-AR, which remains at the membrane. In contrast to desensitization, downregulation denotes a chronic process, during which agonist overstimulation promotes increased receptor degradation concomitant with a reduced *de novo* synthesis rate that does not match the loss of receptors.[12 71]

(iii) Recent reports on homo- and heterodimerization between receptor subtypes suggest a potential concentration of receptor complexity that could account for previously unexpected pharmacological diversity.[3] α_2-AR and β_2-AR may form homodimers. The β_2-AR was found to form dimers on agonist activation, and the agonist-induced homodimer seems to represent the active β_2-AR species. Dimerization is established between the sixth and seventh transmembrane domains of the two receptors involved.[78] Heterodimers have been found between the α_{2A}-AR and β_1-AR, the β_1-AR and β_2-AR, and the α_{2C} and M3-muscarinic receptors, as well as between the β_2-AR and δ-opioid receptors.[3] Formation of the β_1-AR and β_2-AR heterodimer inhibits the agonist-promoted internalization of the β_2-AR and its ability to activate the MAPK cascade[46] (see next section).

(iv) A GPCR-associated protein may directly mediate signalling, as in the case of the G-proteins themselves. Alternatively, a GPCR-associated protein may regulate receptor signalling by controlling receptor localization and/or trafficking, e.g. by internalization. Finally, a GPCR-associated protein may act as a scaffold, physically linking the receptor to various effectors. Scaffold proteins are defined as proteins that associate with two or more partners to enhance the efficiency and/or specificity of cellular signalling pathways. The family of PKA-anchoring proteins (AKAPs) was one of the first to be recognized as scaffold proteins.[30 85 87] Two AKAPs, AKAP250, also known as gravin, and AKAP79, were found to interact with the C-terminus of the β_2-AR.

Relation of adrenergic to global cardiomyocyte signalling

The G-protein-stimulated signal pathways continue via distinct classes of protein serine–threonine kinases (PKA, PKB, PKC, PKG), which specifically phosphorylate serine and threonine residues in their target proteins.[13 60 61 76 85 87 99]

PKA and PKC are the key players in the adrenergic signal relay system. In the absence of cAMP, PKA is an enzymatically inactive tetrameric holoenzyme consisting of two catalytic subunits (PKAC monomer, 40.4 kDa) bound to a regulatory subunit dimer (PKAR monomer, 42.5 kDa). cAMP binds cooperatively to two sites on each PKAR subunit. Upon binding of four molecules of cAMP, the enzyme dissociates into a PKAR dimer with four molecules of cAMP bound, and two free, active PKACs. The PKC exists in a variety of isoforms with functional specificities. There are 10 mammalian PKC isoforms, ranging in molecular weight from 68 to 102 kDa. The four conventional PKCs (α, β1 with a splice variant β2, and γ) require Ca^{2+} and DAG for activation; the four novel PKCs (δ, ε, η/L and θ) are Ca^{2+}-independent but require DAG for activation; finally, the two atypical PKCs (ζ and λ) lack both the Ca^{2+} and the DAG binding domain.

G-proteins are also able to activate the monomeric GTPase Ras via different phosphorelay systems. Ras represents a master switch in transferring extracellular signals for growth and differentiation via the four MAPK cascades to the nucleus (Fig. 2).[33 37 53 55 64] Among these four cascades, (i) ERKs (extracellular signal-regulated kinases) are activated by growth factors and regulate cardiac hypertrophy and apoptosis, (ii) the more recently characterized big MAP kinase-1 (BMK1, also called ERK5) pathway transmits oxidative stress signals to the cell nucleus, (iii) the four p38 MAPK isoforms (α, β, γ and δ) are activated by cytokines and environmental stress and are also involved in regulation of apoptosis, and (iv) the JNKs (c-Jun N-terminal kinases) are critical regulators of transcription. Specificity of AR subtype signalling may be achieved by proteins that do not have intrinsic catalytic activity but serve as adapter and anchoring proteins. By keeping the reaction partners of a particular signalling pathway in close proximity to the effector site, they form so-called signalling modules. Such a signalling module of activated PKCε with ERK has been demonstrated to be operative in mitochondria, where it induces cardioprotection.[5] Another recent example is the conserved sequential MAPK cascade Raf–MEK–ERK, in which the two upstream kinases of the module, Raf and MEK, remain cytoplasmic. In resting cells, ERK is anchored to MEK, whereas upon activation it rapidly detaches and translocates to the nucleus.[73] A similarly complex signalling network with redundancies, as revealed between the downstream signalling pathways of ARs (Fig. 1), can also be discerned among the MAPK cascades (Fig. 2).

Calcium as a signal transmitter

Calcium is highly toxic for any cell, yet it represents the major intracellular messenger regulating most activities, including contractility, metabolism, transport, secretion and transcription. The Ca^{2+} signal varies in its character depending on the circumstances. One may see a long-lasting cytoplasmic increase in Ca^{2+} concentration activating metabolism as well as gene expression, or short-lasting (50–200 ms) Ca^{2+} transients triggering contraction.[7 8 14] Calcium signalling for different cellular responses and its consequent requirement for energy production depend on the strictly maintained intracellular Ca^{2+} homeostasis, which is largely monitored by adrenergic signalling pathways (Fig. 3). The heart makes up less than 0.5% of the body mass, yet it consumes around 11% of the total energy expenditure. The complex scheme in Fig. 3 comprises the explicitly reported interconnections between the intracellular adrenergic and Ca^{2+} signalling pathways, but in reality probably many more may exist. In the resting myocyte the cytoplasmic Ca^{2+} concentration is below 10^{-7} M and increases transiently by almost two orders of magnitude upon stimulation by the action potential (AP). The Ca^{2+} concentration in the mitochondrial matrix follows closely that of the cytoplasm, involving the regulation of metabolic key enzymes of the tricarboxylic acid cycle.[14 99] Several ATP-consuming ion pumps and electrogenically driven ion exchangers are responsible for ensuring a sufficiently low cytoplasmic Ca^{2+} concentration during diastole. In diastole, the cytoplasmic Ca^{2+} concentration must be significantly lower than what is found in ultrapure bidistilled water after passage through an ion exchange resin, as it is used in the laboratory. Any cell damage which impairs the tight control over the cytoplasmic Ca^{2+} concentration leads to abnormal energy metabolism in the mitochondria and ends in cell death.

In contrast to the low cytoplasmic Ca^{2+} during rest, the Ca^{2+} concentration outside the myocyte ranges between 1 and 2 mM, thus establishing a gradient of over 10 000-fold. This outside–inside gradient enables the Ca^{2+} ion to function as a signal, provided there are sufficiently sensitive Ca^{2+}-binding signalling components in the cell.[27] Inside the cardiomyocytes, the Ca^{2+} signal is conveyed to the target sites by the Ca^{2+}-sensing proteins troponin-C (TnC) and calmodulin (CaM), which reversibly bind Ca^{2+} ions with affinities in the range of 10^{-5} to 10^{-6} M. These Ca^{2+} affinities are just in the range attained by Ca^{2+} signalling transients, taking into account the intracellular presence of around 1 mM Mg^{2+} ions, which are competing for the Ca^{2+} binding sites with an affinity 10^4 times lower than that of Ca^{2+}. Some Ca^{2+}-regulated targets have their own Ca^{2+} binding sites and are not dependent on TnC or CaM as an intermediate signal component.

Calcium enters the cardiomyocyte down the concentration gradient via the high voltage-activated (opening at around –20 mV) L-type Ca^{2+}-channels of the sarcolemma (particularly accumulated in the transverse T-tubules) as often as an AP stimulates the cell. The low voltage-activated (opening between –60 and –40 mV) T-type channels are not concentrated in the T-tubules and contribute little to the Ca^{2+} entry from outside.[6 18 34 41] They are primarily found in secretory and smooth muscle cells and in the cardiac nodal cells, where they are involved in rhythm control. The

L- and T-type channels belong to a family of cell surface Ca^{2+}-channels composed of four subunits in a 1:1 stoichiometry ($\alpha1$, $\alpha2$, β, δ). The human $\alpha1C$ (Cav1.2) subunit (2221 amino acids, 249 kDa), occurring in heart (Cav1.2a) and smooth muscle (Cav1.2b sharing 93% homology), contains four domains with six transmembrane helices each (S1–S6, yielding a total of 12 transmembrane helices). The S6s of each domain together form the Ca^{2+} pore. The four transmembrane S4 helices of 19 amino acids each contain positively charged residues at every third position, together forming the voltage sensor of the pore. The P-loop between the transmembrane helices S5 and S6 is very much conserved among the different Ca^{2+}-channels and provides the filter selectivity for Ca^{2+}. The β-subunit associates with the α-subunit at the inner side of the membrane and determines the kinetics of the channel activities (opening, closing, inactivation). The β_2-subunit (73.5 kDa) is characteristic of the heart while the β_3-subunit (54.5 kDa) is more typical of smooth muscle. The Cav1.2 channel activity is enhanced by phosphorylation by PKA, PKC and CaMK, but is inhibited by Ca^{2+} when its cytoplasmic concentration is increased during sustained cell activity (negative feedback control).[34 39 41 93] Calcium binds directly to the C-terminal intracellular part of the Cav1.2, which contains a Ca^{2+}-binding EF-hand domain (see below), but nearby is also a binding site for CaM, which contributes to sensing Ca^{2+} signalling. For prolonged elevation of cytoplasmic Ca^{2+}, ill-defined ligand-gated channels (also called store-operated channels) are thought to be involved in Ca^{2+} entry after the Ca^{2+} release from internal stores.[14 22]

For excitation–contraction coupling, Ca^{2+} is primarily released from intracellular stores in response to extracellular stimuli (Fig. 3). The endoplasmic reticulum (ER) in non-muscle cells and its derivative, the SR in striated muscles (cardiac and skeletal) as well as in smooth muscle, are able to accumulate Ca^{2+} up to a concentration of 30 mM and to store it bound to proteins with multiple low-affinity Ca^{2+}-binding sites, such as calsequestrin and calreticulin. Calsequestrin (46.4 kDa) is found in cardiac and skeletal SR while calreticulin (45.0 kDa) is mainly present in the SR of smooth muscle and ER of non-muscle cells. Both the SR and ER contain two ligand-gated Ca^{2+} release channels, the ryanodine binding receptor (RYR) and the IP3 binding receptor (IP3R). The Ca^{2+} entering the cell on stimulation induces a far larger (5- to 20-fold) release of Ca^{2+} from the closely positioned intracellular SR into the cytoplasm via the RYR, a process called Ca^{2+}-induced Ca^{2+} release (CICR).[7 14 16]

The RYR channel forms a tetramer with four equal subunits of 565 kDa each, which combine with four regulatory proteins, called FKBP12.6. FKBP, with a mass of 12.6 kDa belongs to the group of cyclophilins, which accelerate protein folding, acting as peptidyl-prolyl *cis-trans* isomerases or rotamases. They are inhibited by the immunosuppressor drugs FK506 (tacrolimus) and rapamycin (sirolimus), but not by cyclosporin A. Of the three isoforms, RYR1 (with a total molecular weight of around 2300 kDa) occurs in skeletal muscle, RYR2 in cardiomyocytes and RYR3 in non-muscle cells.[49 88] In addition, the RYR2 serves as scaffold protein, combining with numerous key regulatory components in the junctional SR complex, including CaM, PKA, type-1 and type-2 phosphatases and, at the luminal SR surface, triadin and calsequestrin. Calcium can also be released from the SR via the IP3R channel, which is composed of four equal subunits of 313 kDa each. The IP3R channel also exists in three isoforms, with IP3R2 in cardiomyocytes. The IP3R2 is activated by IP3 produced by PLC. The rate and extent of Ca^{2+} liberation by IP3 is much lower than for CICR via the RYR2, and thus hardly contributes to excitation–contraction coupling in cardiomyocytes. However, intracellular Ca^{2+} release by IP3 is important in the slow motion of smooth muscle contraction and in fine-tuning the activity of atrial myocytes, where the SR has more IP3R2 than in the ventricular myocytes. RYR2 and IP3R2 share structural and functional similarities and have some sequence similarity in their C-terminal domains, although the latter is about half the size of the former.[77] The two Ca^{2+} release channel types interact and are inhibited by high Ca^{2+} and CaM, but become activated by phosphorylation by PKA, PKC or a Ca^{2+}-CaM-dependent protein kinase-II (CaMK-II) (Fig. 3). Phosphorylation of RYR2 induces dissociation of the FKBP regulatory protein, which inhibits the RYR2 channel when bound to it.

Intracellular calcium handling in cardiomyocytes

Cardiomyocyte contraction and cell activity in general are terminated by removing Ca^{2+} from its regulatory sites on TnC, CaM and other proteins. This is achieved by quickly and efficiently lowering the cytoplasmic Ca^{2+} concentration.[8 14 27 45] Two proteins are involved in moving Ca^{2+} out of the myocyte, the sarcolemmal Ca^{2+} pump (PMCA) and the Na^+/Ca^{2+}-exchanger (or antiporter, NCX1), and one in taking it up into the intracellular store (the Ca^{2+} pump of the SR, SERCA2a) (Fig. 3). The immediate removal of Ca^{2+} from the regulatory sites is effected by the SERCA2a pump, which has high affinity but low transport capacity. The NCX1 extrudes Ca^{2+} out of the cell during diastole with relatively low affinity but high transport capacity, thus ensuring a sufficiently low Ca^{2+} concentration during cell rest. While the NCX1 couples the outward transport of one Ca^{2+} to the entry of three Na^+ down its outside–inside gradient, the SR and the sarcolemmal Ca^{2+} pumps depend on ATP consumption. The outside–inside Na^+ gradient (established by the Na^+/K^+ pump, which also uses ATP) allows the electrogenically driven exchange of Ca^{2+} against Na^+ by the NCX1. Stimulation of the Na^+/K^+ pump by phosphorylation by PKA and/or PKC is under the control of the ARs, in particular the β_1-AR (Figs 2 and

3). In mice and rats, about 92% of the Ca^{2+} ions are pumped back into the SR by the SERCA2a and only 7% are extruded out of the cell by the NCX1. In larger mammals, such as rabbits, cats, dogs and humans, SERCA2a and NCX1 are able to lower the Ca^{2+} concentration to about 70 and 28% respectively. The slow sarcolemmal PMCA pump contributes little (1–2%) to the process of lowering the cytoplasmic Ca^{2+} concentrations in the heart, whereas its function is decisive for Ca^{2+} homeostasis in smooth and non-muscle cells.

The amount of AP-induced Ca^{2+} entry is primarily dictated by the duration of the AP and the open probability of the L-type Ca^{2+}-channel. The Ca^{2+} extruded after the heart-beat must match the amount of Ca^{2+} that enters just before the beat, otherwise the cell would not be in steady state but would either lose or gain Ca^{2+}. This provides a quantitative framework for the dynamic Ca^{2+} fluxes in the cardiomyocytes. The SERCA2a pump is one of the main players in terminating contraction and restoring resting cytoplasmic Ca^{2+} concentrations. It is known that during heart failure in humans, as well as in a rabbit model, the functional expression of SERCA2a is reduced and NCX1 is increased.[8 32] This results in a loss of SR Ca^{2+} concomitant with enhanced Ca^{2+} extrusion, leading to a net loss of intracellular Ca^{2+}. Consequently, less Ca^{2+} is available in the SR for the subsequent heart-beats, which is the central cause of systolic contractile deficit in heart failure.

The NCX1 (splice variant NCX1.1) is the cardiac isoform with 938 amino acids (104 kDa), whereas the NCX2 is preferentially expressed in the brain and NCX3 in skeletal muscle.[62 70 82 100] Its topology is not yet clear, suggesting either 11 or nine transmembrane helices. Interestingly, the NCX1 may function in reverse mode during the plateau of the AP (Ca^{2+} influx coupled with Na^{+} outflux). This may be of physiological significance as the NCX1, like the L-type Ca^{2+}-channel, is concentrated in the transverse T-tubular system and may be able to elicit Ca^{2+}-induced Ca^{2+} release via the RYR2 from the SR. This would reinforce the Ca^{2+} release process from the RYR2 ascribed to the interaction of the L-type Ca^{2+}-channel with the RYR2 (as mentioned above). The NCX1 can be phosphorylated and stimulated by both PKA and PKC, and is therefore under the control of ARs. PMCA and SERCA belong to a subfamily of the P-type ATPases. The sarcolemmal Ca^{2+} pump (PMCA) transports one Ca^{2+} per ATP out of the cell, while SERCA2a takes two Ca^{2+} per ATP up into the SR. From the isoforms of the four genes giving rise to PMCA1-PMCA4 and over 20 splice variants, PMCA1c (1249 amino acids, 138 kDa, with 10 putative transmembrane helices) seems to be the main cardiac species, but other isoforms are also expressed in cardiomyocytes. PMCA activity depends on binding CaM, and may be further stimulated by phosphorylation via PKA and/or PKC (Fig. 3).

The cardiac SR Ca^{2+} pump SERCA2a, together with its regulatory protein phospholamban (PLN), is the most important component linking adrenergic control to inotropy and rhythmicity.[4 8 14 22 48] Three genes were identified for the SR Ca^{2+} pump, SERCA1, SERCA2 and SERCA3, which are spliced into several isoforms. SERCA1a is mainly expressed in fast skeletal muscle, while SERCA1b is abundant in fetal and neonatal tissues. The SERCA2 gene encodes four splice variants: SERCA2a, expressed in the heart and in slow skeletal muscle, SERCA2b, expressed in smooth muscle and with variants (types 2 and 3) in non-muscle cells and (type 4) in neuronal cells. The recently solved crystal structure of the SERCA1a pump in the Ca^{2+}-bound state reveals (besides the 10 transmembrane helices) three large cytoplasmic domain structures constituting the nucleotide binding site, the catalytic site and the phosphorylation site.[91] The cardiac SERCA2a is 997 amino acids long (110 kDa) and is under the direct control of a CaM-dependent kinase-II (CaMK-II), which enhances its transport capacity by phosphorylation of Ser38. The major regulator of SERCA2a, however, is PLN, with 52 amino acids (6.1 kDa) and one transmembrane helix (C-terminal amino acids 32–52). It is predominantly expressed in ventricular cardiac muscle, but also in small amounts in slow-twitch skeletal muscle, smooth muscle and endothelial cells. As a monomer, it associates with and efficiently inhibits the SERCA2a pump, by interaction of its transmembrane helix with helices of the pump and its cytoplasmic domain with the cytoplasmic domain of the pump. Its inhibitory effect is delicately regulated by phosphorylation induced by different signalling pathways. The cytoplasmic domain is amenable to regulation by phosphorylation of Ser16 by PKA, Thr17 by CaMK-II, and Ser10 by PKC (Fig. 3). Phosphorylation to various degrees causes gradual dissociation of PLN from SERCA2a, relieving the inhibition of the Ca^{2+} pump and thus increasing the rate of relaxation (lusitropic effect). When not associated with SERCA2a, PLN polymerizes into pentamers.

To further increase the complexity, sarcolipin (SLN), which is a homologue of PLN with 31 amino acids (3.8 kDa), forming just one transmembrane helix and lacking most of the cytoplasmic portion present in PLN, was discovered recently.[4 48] It is highly expressed in human fast-twitch skeletal muscle and in small amounts predominantly in atrial muscle. SLN inhibits the SERCA1a (fast skeletal muscle) and SERCA2a (cardiomyocytes) by lowering the Ca^{2+}-binding affinity and slowing the ATPase turnover rate. In addition, SLN is able to induce a superinhibitory effect apparently by binding to PLN and thus preventing PLN from polymerizing into pentamers. A small amount of SLN may be sufficiently potent to shift the equilibrium of pentameric PLN towards the monomer, inhibiting SERCA2a. In view of the decisive power of the SR-SERCA2a pump in regulating contractility, both SERCA2a and PLN represent potential targets for new therapeutic approaches.

Adrenergic fine-tuning of contractility

TnC and CaM are the main transmitters of the intracellular Ca^{2+} signal. Both belong to the EF-hand protein superfamily, which contains 66 distinct subfamilies with a total of almost 600 known proteins.[59] The characteristic EF-hand, with 30 amino acids, consists of a flexible loop (eight amino acids) with five residues, providing oxygen for reversibly complexing with Ca^{2+}, which is flanked on both sides by a short α-helix of approximately 11 amino acids. Reversible binding of Ca^{2+} to the loop induces a conformational change that moves the two rigid helices relative to one another, and this movement is transmitted to the associated target proteins. Both TnC and CaM contain four such Ca^{2+}-binding domains, which have arisen from a common single Ca^{2+} binding ancestor protein by several steps of gene duplication and fusion. The expression of TnC is restricted to striated muscles (skeletal and cardiac), where it regulates contraction and is located in the heterotrimeric troponin complex on the actin filament in the sarcomeres. This complex consists, besides TnC, of troponin-I (inhibitory component, TnI) and troponin-T (tropomyosin binding component, TnT).[51 66 67] TnC contains either two ions or two ions bound to the two C-terminal binding sites for protein stabilization, whereas in the fast skeletal muscle isoform (159 amino acids, 18.0 kDa) the two N-terminal sites reversibly bind signalling Ca^{2+}.[27 59 79] In the cardiac TnC (TnC) isoform (161 amino acids, 18.4 kDa) only one of the two N-terminal binding sites is functional for regulation. Binding of Ca^{2+} to the regulatory site of cTnC modulates its interaction with TnI and, as a consequence, it also affects the interaction of TnI with TnT and tropomyosin. In the absence of Ca^{2+}, TnI inhibits the interaction of myosin-II with actin (relaxation). Binding of Ca^{2+} to cTnC relieves the inhibitory effect of TnI, and contraction ensues. The troponin complex is positioned along the actin filament at every seventh actin monomer, and the Ca^{2+}-induced conformational changes of TnC are imparted to all actin monomers via TnI and TnT (around 32 kDa) acting through the two tropomyosin threads that run on both sides along the entire actin filament (Fig. 4). Tropomyosin is an elongated dimeric protein about 40 nm long, comprising two α-helical subunits (284 amino acids each, 32.8 kDa) arranged in parallel orientation. The tropomyosin molecules are strung together head-to-tail, thus forming threads along the actin filaments with a length of 600–1000 nm. Cardiac TnI (210 amino acids, 24.0 kDa) is somewhat larger than its skeletal muscle counterparts and includes a unique 32-residue N-terminal extension with two adjacent residues, Ser23 and Ser24, that can be phosphorylated by either PKA or PKC. TnI phosphorylation under the control of distinct AR subtypes (Fig. 3) lowers the affinity of TnC for calcium ions. Calcium then more readily dissociates from TnC at the end of systole, thus enhancing relaxation (lusitropic effect).

In contrast to TnC, CaM (148 amino acids, 16.7 kDa) is an ubiquitously expressed Ca^{2+} signalling protein found in all

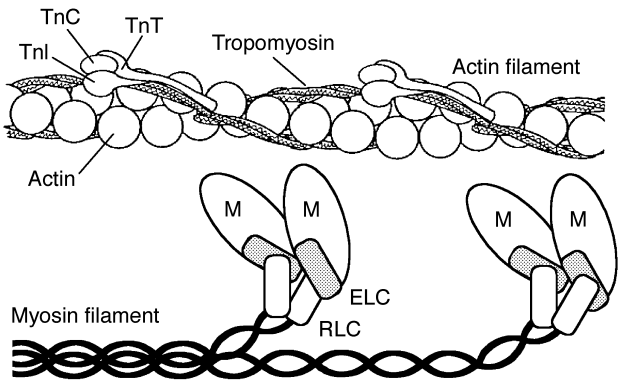

Fig 4 Scheme of sarcomeric contractile and regulatory proteins. M=pairs of myosin heads forming the contractile crossbridges (myosin tails are black); ELC=essential myosin light chains; RLC=regulatory myosin light chains; TnC, TnI, TnT=troponin components associated with the tropomyosin threads. Upon binding of Ca^{2+} to TnC, the troponin complex moves the tropomyosin threads deeper into the groves along the actin filament so that the myosin heads can attach to the actin monomers and contract. In the absence of Ca^{2+} the tropomyosin threads prevent the myosin heads from attaching to actin (relaxation, steric blocking model).[51 57 79]

eukaryotic cells. Its amino acid sequence is identical in the human, rat, mouse, rabbit, chicken, frog, sea urchin and torpedo fish.[59 77] This makes it one of the most highly conserved proteins known. With four Ca^{2+} bound, it regulates over 40 enzymes, channels and structural proteins. In the so-called apo form, the Ca^{2+} binding sites are empty or may be occupied by Mg^{2+}. It operates by (i) reversible binding to the target protein when Ca^{2+} is bound, (ii) remaining combined with some proteins in both apo and Ca^{2+}-bound form, as in the heteromeric phosphorylase complex, or (iii) forming part of the target protein structure as heterochimera, the gene encoding EF-hands being fused to the gene of the target protein. While Ca^{2+}-CaM interacts with PMCA, L-type Ca^{2+}-channel and RYR2 (as mentioned above) for direct regulation, most of its regulatory activities are mediated by CaM-dependent kinases (CaMKs) and subsequent phosphorylation reactions (Fig. 3). CaMKs are activated by phosphorylation, like most protein kinases, either by autophosphorylation triggered by binding of CaM or by another CaM-dependent kinase, called CaMK kinase (CaMKK). The role of this CaMKK is reminiscent of that of MAP kinase kinase (MAPKK) in the MAP kinase cascade (Fig. 2).[38 44] Several types of CaMK are multisubstrate kinases that phosphorylate different target proteins, while others are specific for one particular protein; for instance, the myosin light chain kinase (MLCK). The multisubstrate CaMK-II is a multimeric enzyme composed of up to 12 subunits, whereas the skeletal and cardiac muscle MLCK (65 kDa) is monomeric. The MLCK phosphorylates specifically the regulatory light-chain subunit (RLC) of cardiac myosin-II at Ser14, rendering the contractile apparatus more sensitive to the Ca^{2+} trigger (positive inotropy). The same can also be achieved by RLC phosphorylation by PKC (Fig. 3).

Frank–Starling and negative feedback mechanisms

Physiological contraction generates both isometric force (ventricular pressure) and rapid shortening to eject the blood. As discussed above, there are two main ways to change the strength of contraction: by altering the amplitude and/or duration of the Ca^{2+} transient, and by altering the sensitivity of the contractile filaments to Ca^{2+} (by phosphorylation of the myosin RLC and/or TnI). Calcium-sensitivity also increases by mechanical stretching as the heart fills with blood, resulting in stronger subsequent contraction.[23] This is due to the compression of the transverse filament lattice that occurs on stretching and brings the contractile filaments in the sarcomeres closer together. This facilitates actin–myosin interaction and increases the affinity of TnC for Ca^{2+}. It represents an important autoregulatory mechanism by which the heart adjusts to increased diastolic filling, and is called the Frank–Starling response.

In general, the positive inotropic and lusitropic effects are mainly elicited by β_1- and β_2-AR stimulation and mediated through cAMP-dependent PKA phosphorylation of PLN, TnI, the L-type Ca^{2+}-channel and RYR2. The degree of positive inotropy is directly related to the amount of cellular Ca^{2+}, which steadily increases with time under these conditions. Without safety precautions, it would end in cardiac arrest in systolic contracture. This is, however, normally not observed (except with digitalis glycoside overdoses), as the cardiac phosphodiesterase (PDE) isoforms degrade cAMP, thus keeping the overall β_1-AR stimulation at bay. At least four types of PDE isoforms (PDE1–PDE4, varying in size from 61 to 124 kDa), with over 20 splice variants, most of them membrane-associated, have been identified in cardiomyocytes.[25 35 52 83] Via cyclic nucleotide metabolism, PDEs are also involved in the regulation of the L-type Ca^{2+}-channels. The activity of PDE1 depends on the association of the four Ca^{2+} bound form of CaM, which increases concomitantly with the increase in cytoplasmic Ca^{2+}; PDE2 is stimulated by cAMP; PDE3 is inhibited by cAMP; and PDE4 is insensitive to cAMP. The PDEs are operative as homodimers and contribute decisively to the contractile responsiveness. The fact that PDE activity reduces inotropy by degradation of cAMP prompted the development of PDE inhibitors, such as amrinone, milrinone and enoximone (bipyridines inhibit PDE enzymes, as do methylxanthines), which are in clinical use for the acute treatment of congestive heart failure. Despite its complexity, the PDE system provides a powerful negative feedback control system of great physiological significance (Fig. 3).

In conclusion, although Ca^{2+} is traditionally described as a second messenger that is liberated from intracellular stores (SR), Ca^{2+} entering the cell may activate a number of processes acting directly as a first messenger.[14] In particular, it amplifies its own signalling capacity by the Ca^{2+}-induced Ca^{2+} release from the SR via the RYR2, thus also acting as a second messenger. Furthermore, the second messenger IP3, liberated by PLC from PIP2 (as mentioned above), provokes the release of Ca^{2+} via the IP3R of the SR, acting then as a third messenger. In addition, adrenergic signalling pathways exert their effects (balancing between positive or negative inotropy and lusitropy) almost exclusively by modulating cytoplasmic Ca^{2+} concentrations and signalling transients in amplitude and frequency, thus adding yet another step to the Ca^{2+} signalling cascade. Consequently, Ca^{2+} may well be viewed as operating at the same time as a first, second and third messenger. In the healthy heart, the combined signalling circuits coordinate contractility and energy production in a concerted way. The cellular and molecular basis explains how adrenergic stimulation induces positive inotropy together with positive lusitropy (faster relaxation) under increased haemodynamic load. This combination of stronger but shorter contraction twitches makes it possible to accommodate more beats per time interval, thus increasing cardiac output under increased workload. However, upon transition from compensated haemodynamics to overt heart failure or during ischaemia/reperfusion injuries, the integrated signalling network may become unbalanced and dysfunctional, eventually ending in collapse.[32 49 71 79 99]

Adrenergic regulation of heart rate

Maintaining adequate perfusion of vital organs is the main task of the myocardium. Adaptation to increased workload is effected by coordination of two principal mechanisms, which both are under sympathetic and parasympathetic control: (i) positive inotropy as described above, and (ii) increase in heart rate. Recently a voltage-gated cation channel family was cloned comprising four members: HCN1–HCN4, in the 89–129 kDa range.[9 40 56] HCN channels open upon hyperpolarization and close at positive potentials. cAMP and cGMP increase channel activity by shifting the activation curve of the channels to more positive voltages. The stimulatory effect of cyclic nucleotides is not dependent on protein phosphorylation but is due to a direct interaction with the HCN channel protein. HCN channels contain six transmembrane helices (S1–S6) and are believed to assemble in tetramers. The S4 segment is positively charged and serves as voltage sensor. The C-terminal region of all HCN channels contains a cyclic nucleotide binding domain that confers regulation. The channels are activated during membrane hyperpolarization after the termination of an action potential and provide an inward Na^+ current that slowly depolarizes the plasma membrane. Sodium permeates the channels five times more readily than K^+. HCN channels are found in neurons and heart cells. HCN4 represents the predominant species in the sino-atrial node (SAN) controlling heart rate and rhythm. HCN2 is the most abundant neuronal channel and is found almost ubiquitously in the brain. Sympathetic stimulation of the SAN raises

cAMP concentrations, thus accelerating diastolic depolarization and heart rate. Stimulation of muscarinic acetylcholine receptors slows the heart rate by the opposite action (Fig. 1). Given the key role of HCN channels in cardiac pacemaking, these channels represent promising pharmacological targets for the development of novel drugs for the treatment of cardiac arrhythmias and ischaemic heart disease.

Regulation of vascular smooth muscle contraction

In cardiomyocytes, adrenergic signalling by β_1- and β_2-AR causes robust positive inotropy. In contrast, the main β_2-AR signalling in bronchial and vascular smooth muscle cells induces relaxation. This also applies to the contractility and motility of non-muscle cells in general. Nevertheless, in both cardiomyocytes and in smooth muscle cells the β-AR signalling operates primarily via Gαs–AC–cAMP–PKA (Fig. 1). The difference resides in the regulatory mechanism of the non-sarcomeric contractile apparatus, in which the myosin-II and actin filaments are not so regularly arranged as in the sarcomeres of striated muscles. Smooth muscle myosin-II is similar to its counterpart, consisting of two heavy chains (HC, around 200 kDa) and two types of light chains (in the 20 kDa molecular weight range). The N-terminal portion of the HC folds into the globular myosin head that forms the motor domain (Fig. 4).[57 79] The two myosin heads together form the crossbridge, which displaces the actin filament by cyclic interactions that convert chemical energy obtained from the hydrolysis of ATP into mechanical movement. The C-terminal half of the HC forms an α-helix and the two helices of one molecule together form a left-handed superhelix, called the myosin tail. The myosin tails associate together and form the myosin filament. One essential light chain and one regulatory light chain (RLC) are non-covalently associated with each myosin head (Fig. 4). The enzyme kinetics and rates of crossbridge movement are significantly slower for smooth muscle than for cardiac myosin-II.

The actin filaments do not contain troponin, yet smooth muscle contraction is still regulated by Ca^{2+}, albeit in an indirect way.[38 50 57 86] The on–off switch for contraction depends on the phosphorylation of smooth muscle myosin RLC at Ser19 by the Ca^{2+}-CaM-dependent smooth muscle MLCK isoform and dephosphorylation by a myosin light chain phosphatase (MLCP) (Fig. 5). The extent of RLC phosphorylation determines shortening velocity and tension development in smooth muscle. A second phosphorylation of RLC at the adjacent Thr18 has only a small additional positive effect on ATPase activity. Alternatively, the RLC can also be phosphorylated by PKC, CaMK-II and other protein kinases. In addition, contraction and relaxation of smooth muscle may also be induced by hormones and cell

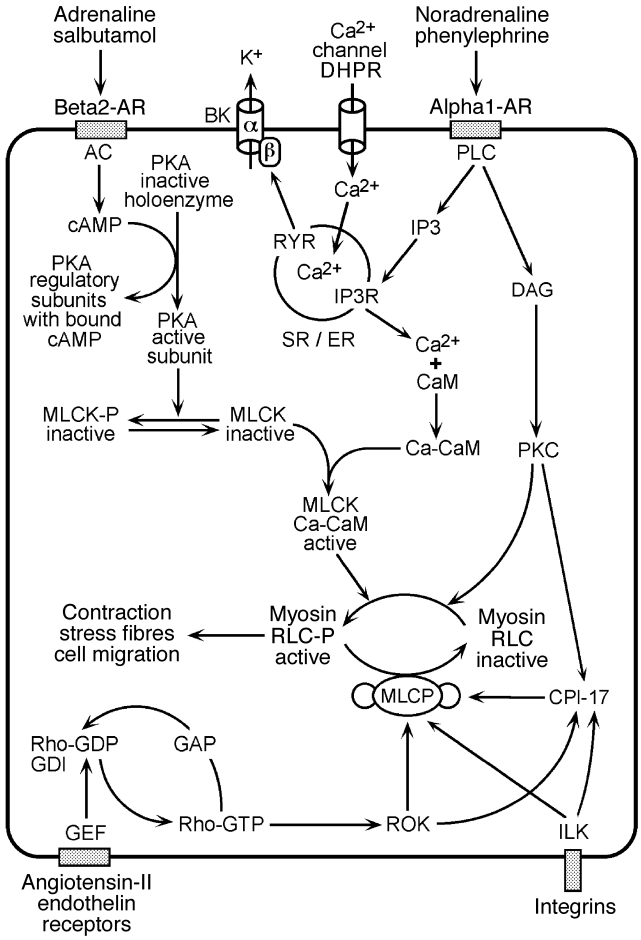

Fig 5 Regulation of contraction in smooth muscle and non-muscle cells.[10 15 17 31 38 41 50 57 75 81 84 86 89 95] AC=adenylyl cyclase; AR=adrenergic receptor; BK=large-conductance Ca^{2+}-activated potassium channel; CaM=calmodulin; cAMP=cyclic AMP; CPI-17=MLCP inhibitor; DAG=diacylglycerol; DHPR=dihydropyridine receptor (L-type Ca^{2+}-channel); GAP=GTPase-activating protein; GDI=GDP dissociation inhibitor; GDP=guanosine diphosphate; GEF=GTP exchange factor; GTP=guanosine triphosphate; ILK=integrin-linked kinase; IP3=inositol trisphosphate; IP3R=IP3 receptor (SR Ca^{2+} release channel); MLCK=myosin light chain kinase; MLCP=myosin light chain phosphatase; PKA, PKC=target-specific serine–threonine protein kinases; PLC=phospholipase C; Rho=small monomeric GTPase; RLC=myosin regulatory light chain; ROK=Rho-dependent kinase; RyR=ryanodine binding receptor (SR Ca^{2+} release channel). Note that MLCK requires Ca-CaM for activation (phosphorylation may inhibit CaM binding), whereas MLCP is inactivated by phosphorylation of the large myosin-targeting subunit.

mediators, bypassing the canonical Ca^{2+} signalling pathways (see below).

The myosin RLC is the only known physiological substrate for the MLCK.[38 86] In vertebrates there are two genes for MLCK. The skeletal muscle gene encodes a kinase (65 kDa) containing a catalytic core and a regulatory domain with an autoinhibitory and CaM binding sequence. The smooth muscle gene expresses three transcripts in a cell-specific manner because of alternate promoters and splice variants, one with a molecular weight of 130 kDa, the

second with 210 kDa, and the third consisting of only the C-terminal immunoglobulin-like domain of 17.5 kDa (lacking the catalytic and regulatory domains), called telokin. The short species (130 kDa) predominates in smooth muscle while the long isoform (210 kDa) occurs in many different non-muscle tissues and to a lesser extent also in smooth muscle cells. Telokin is mainly found in phasic smooth muscle tissues and may contribute to Ca^{2+} desensitization by cyclic nucleotides. The MLCK isoforms of smooth and non-muscle tissues contain in their N-terminal region several immunoglobulin-like domains, one fibronectin-like domain, and an actin-binding sequence, all of which are not present in the skeletal muscle MLCK. When Ca^{2+} binds to CaM, it associates with MLCK, resulting in its activation and phosphorylation of the RLC (Fig. 5). At this point, catecholamine signalling via β2-AR is connected to the activation process of smooth muscle contraction, allowing relaxation even in the presence of activatory Ca^{2+} concentrations. Phosphorylation of MLCK by cAMP-dependent PKA at the C-terminal Ser512 of the CaM binding sequence lowers the affinity for Ca^{2+}-CaM about 10-fold, thus resuming its inactive state by auto-inhibition of its regulatory domain. Like the myosin RLC, the MLCK can be phosphorylated by different kinases in addition to PKA, such as PKC, CaMK-II and PAK (p21-activated protein kinase) at various sites.

It was first thought that regulation of smooth and non-muscle contractility resides primarily in the activatory process of RLC phosphorylation, followed by a more or less constant rate of dephosphorylation by a constitutively active MLCP (thought to represent a housekeeping enzyme). More recently it was recognized that the MLCP also is regulated by phosphorylation.[84 86] MLCP is a holoenzyme consisting of three subunits: a catalytic subunit of 37 kDa (PP1c), a large myosin binding subunit (MBS) of around 130 kDa (also known as myosin phosphatase targeting subunit, MYPT), and a small 20 kDa subunit (M20) of unknown function. The PP1c is a member of the type-1 protein serine–threonine phosphatase family. The MBS exists in two splice variants, M130 and M133. MBS binds the relatively non-specific phosphatase PP1c near its N-terminus and confers substrate specificity by forming a complex with myosin-II. MBS preferentially associated with myosin-II when the RLC is phosphorylated and ready for dephosphorylation. M20 binds to the N-terminal portion of MBS. Inhibition of MLCP by phosphorylation at Thr655 in M130 (amino acid numbering for the human species) or Thr696 in M133 by Rho-kinase-II (ROK-II) leads to sustained vasoconstriction without concomitant lowering of the cytoplasmic Ca^{2+} concentration (Fig. 5). Phosphorylation of MBS induces dissociation from myosin-II and from the catalytic PP1c subunit. Additional phosphorylation sites for several different types of kinases have also been shown for MBS *in vitro*, but their physiological significance has not yet been established. Dephosphorylation of MBS proceeds slowly and does not allow fast relaxation of the contractile apparatus.

However, another small molecular weight (16.7 kDa) protein, CPI-17, is a potent reversible inhibitor of MLCP.[84 86] The inhibitory activity of CPI-17 is increased more than 1000-fold by phosphorylation at Ser38 by PKC, PKA, PKG or ROK, and also quickly reversed upon dephosphorylation by the protein serine–threonine phosphatases PP2A, PP2B and PP2C, but not by type-1 protein phosphatases. Consequently, CPI-17 is considered to act in concert with myosin RLC phosphorylation by MLCK as the on–off switch of smooth muscle contraction.

Phasic contraction and vascular tone

The Rho–ROK signalling pathway represents a sophisticated device for fine-tuning vascular tone subject to a variety of agonists, including noradrenaline, angiotensin-II, endothelin, vasopressin, thrombin, prostanoids and many others.[75 84 86 89 95] All these agonists operate via Gαq-coupled receptors that activate both Rho and PLC. The PLC pathway, besides activation of PKC by DAG, produces sufficient IP3 to elicit a short-lasting Ca^{2+} transient by release of Ca^{2+} from the SR via the IP3R channel (Fig. 5). This results in myosin RLC phosphorylation by MLCK and induction of a phasic contraction followed by a long-lasting increase in tone. The Rho–ROK pathway sets the tone of the contractile system by Ca^{2+}-independent mechanisms, preventing dephosphorylation of the myosin RLC. However, this regulation is only operative at a cytoplasmic Ca^{2+} concentration moderately higher than during complete relaxation, maintaining partial activation of the MLCK and a moderate degree of RLC phosphorylation.

The Rho protein family comprises a group of small monomeric GTPases (around 20 kDa) that function as pivotal regulators in cell motility, cell proliferation and apoptosis, involving the actin cytoskeleton and the microtubule network. RhoA with bound GDP is kept in the cytoplasm by association with GDI (GDP dissociation inhibitor). The activity of RhoA is governed by the two factors GEF (GTP exchange factor) and GAP (GTPase activating protein). Agonist binding to surface receptors induces the receptor-coupled Gαq to activate GEF, which in turn promotes dissociation of GDI from RhoA and its exchange of GDP for GTP (Fig. 5). RhoA with bound GTP associates with ROK, releasing its autoinhibitory peptide loop and the activated ROK then inhibits the MLCP by phosphorylation of the MBS subunit. Subsequently, the myosin RLC remains partially phosphorylated, maintaining vascular tone. ROK is a protein serine–threonine kinase of around 160 kDa with two isoforms: ROK-I (ROKβ) and ROK-II (ROKα). ROK-II is the main species in muscles and brain. Besides the activation by RhoA, its activity can be modulated further by autophosphorylation. In addition to inhibition of the MLCP (by phosphorylation of MBS),

ROK-II is also able to phosphorylate the myosin RLC and thus contributes in two ways to the maintenance of vascular tone.

Another mechanism contributing to the vascular tone is the integrin-linked kinase system (Fig. 5).[36 89] Integrins are a family of cell adhesion receptors composed of an α-subunit (several types of 140–210 kDa) and a β-subunit (several types of 90–130 kDa), which are involved in establishing cell-to-cell and cell-to-extracellular matrix (ECM) contacts. Integrins are potential force-transduction proteins because they span the surface membrane and link the ECM to the underlying actin cytoskeleton in specialized focal contact regions. Integrins activate signalling cascades similar to those activated by growth factor receptors (Figs 1 and 2), but unlike these, integrins possess no intrinsic tyrosine kinase activity and must therefore signal via cytoplasmic kinases (Fig. 5). Such an integrin-linked serine-threonine kinase (ILK) has recently been shown to phosphorylate the MBS of MLCP, CPI-17, as well as the myosin RLC in a Ca^{2+}-independent manner. Induction of Ca^{2+}-independent contraction by RLC phosphorylation by ILK becomes apparent only when the MLCP is inhibited. Whether additional tyrosine kinases upstream of ILK are involved in this signalling pathway is not known at present.

Further fine-tuning of smooth muscle contraction is brought about by regulatory mechanisms at the actin filaments.[50 58 95] The regulatory protein caldesmon (93.3 kDa) is complexed to actin, tropomyosin and a Ca^{2+}-binding protein (CaBP). In the absence of Ca^{2+}, caldesmon restricts the interaction of the myosin heads with actin, but when Ca^{2+} is bound to CaBP this inhibition is relieved. CaBP has the same molecular weight as CaM (16.7 kDa) and is believed to represent a covalently modified form of CaM by phosphorylation at Thr79 and Ser81. An additional basic protein, calponin, of 33 kDa, predominantly found in smooth muscle, binds to filamentous actin and CaM. Its role is not clear yet, but it seems to be involved in α1-agonist-induced signal transduction. In particular, it may facilitate the PKC-ERK1/2 signalling pathway, leading to phosphorylation of caldesmon, which also relieves the actomyosin inhibition of the latter.

Intracellular calcium handling in smooth muscle cells

Intracellular Ca^{2+} handling is as tightly controlled in smooth muscle as it is in cardiomyocytes.[10 17 31 41 95] As mentioned above, IP3 induces Ca^{2+} transients by activation of the IP3R release channel in the SR. The activity of the voltage-gated L-type Ca^{2+}-channel with the smooth muscle-specific Cav1.2b subunit is modulated by PKA, PKC, CaM and Ca^{2+} in a similar way to the modulation of the cardiomyocyte species Cav1.2a. For many years it was not clear whether membrane-delimited G-proteins (Gαs and/or Gβγ) might be involved in Ca^{2+}-channel regulation. However, newer experimental evidence rejects this possibility. Interestingly, most of the L-type Ca^{2+}-channels are found in surface membrane caveolae closely packed together with the Na^+–Ca^{2+}-exchanger (NCX1), the Na^+–K^+ pump, the sarcolemmal Ca^{2+} pump (PMCA) and the large-conductance Ca^{2+}-activated potassium channel (BKCa). These caveolae thus assemble the key players in Ca^{2+} handling and are, furthermore, in close proximity to parts of the SR.

The smooth muscle L-type Ca^{2+}-channel mainly serves to fill and maintain the Ca^{2+} content of the SR (Fig. 5). As in myocytes, the Ca^{2+}-channel is activated by PKA phosphorylation induced by β-AR stimulation, increasing cAMP. PKA is recruited to the membrane for Ca^{2+}-channel phosphorylation by the PKA-anchoring protein AKAP. At high concentrations of cAMP, the Ca^{2+}-channel stimulation by PKA may, however, be attenuated for the following reasons. PKG is an important mediator of vascular relaxation induced by the endothelial derived relaxing factor nitric oxide (NO), which stimulates the cytoplasmic guanylyl cyclase (GC) by interaction with its haem group and produces cGMP.[2,47] cGMP activates PKG, which lowers intracellular Ca^{2+} by inhibition of the L-type Ca^{2+}-channel, activates the BKCa (see below), and also activates the MLCP (Fig. 5). Whether inhibition of the Ca^{2+}-channel is achieved by direct phosphorylation of the Cav1.2b subunit or by activation of a protein phosphatase is not clear at present. To complicate matters further, neither cAMP nor cGMP displays absolute specificity for its respective kinase. Thus, some of the actions of cAMP at high concentrations may be due to stimulation of both PKA (channel activation) as well as of PKG (channel inhibition).

One of the most salient features of the caveolae arrangement for the regulation of Ca^{2+} entry into the cell rests on the close juxtaposition with the SR.[10 15 47 81] Calcium entry locally activates the RYR release channel of the SR and thus triggers so-called Ca^{2+} sparks, which in turn activate the BKCa channel in the caveolar vicinity. The BKCa consists of the channel-forming α-subunit (125 kDa) and a small regulatory β-subunit (21.5 kDa). The β-subunit is selectively expressed in smooth muscle, localizes to the intracellular side of the channel, and markedly increases the Ca^{2+}-sensitivity of the channel complex. PKA and PKG seem to stimulate whereas PKC may inhibit the BKCa channel activity. The BKCa is activated either by elevation of the intracellular Ca^{2+} or by diminishing the membrane potential. Together with the voltage-dependent K^+-channels, it is mostly responsible for the membrane or resting potential. The caveolar Ca^{2+} pathway over the short distances from the L-type Ca^{2+}-channel to the SR and from the RYR of the SR back to the sarcolemmal BKCa represents a feedback loop where Ca^{2+} entry leads immediately to a more negative resting potential. This arises by activation of the potassium outflow through the BKCa shielding the cell from undue Ca^{2+} entry. The BKCa seems not to be present in the myocyte surface membrane.

Table 1 Main adrenergic receptor subtype signalling and functions in nervous, cardiac and vascular tissues. CNS, central nervous system; for signalling components consult legends to Figures 1–3. Literature references are given in the text

Adrenergic receptor subtype	Main signalling pathways (compare Figs 1–3, and 5–6)	Adrenergic functions	
β1	Gs–AC–cAMP–PKA	Positive effects on heart rate, inotropy, lusitropy, metabolism, growth, and myocyte toxicity	
β2	Gs–AC–cAMP–PKA, and Gi–PLC–DAG–PKC, and Gβγ–PI3K–PDK1–PKB	Positive effects on inotropy, lusitropy, metabolism, growth, and myocyte survival Presynaptic stimulation of NA release	Relaxation of bronchial and vascular smooth muscle cells
β3	Gi–PLC–DAG–PKC, and Gi–NOS–NO–GC–cGMP–PKG	Negative inotropy, and myocyte survival	
α1A	Gq–PLC–IP3–Ca^{2+}, also Rho–Rho kinase also ERK, JNK, p38	Positive effects on heart rate, inotropy and growth in myocytes	Vasoconstriction
α1B	Gq–PLC–IP3–Ca^{2+}, also ERK, p38 (not JNK)	Positive effects on heart rate, inotropy, and growth in myocytes	Vasoconstriction
α1D	Gq–PLC–IP3–Ca^{2+}, only weakly MAPKs		Mainly in vascular smooth muscle cells, vasoconstriction
α2A	Gi–inhibiting AC	Presynaptically reduces sympathetic outflow at high frequency nerve activity	Lowers blood pressure
α2B	Gi–inhibiting AC	Postsynaptically counteracts the effects of alpha2A	Vasoconstriction in the periphery, in the CNS salt-induced hypertension
α2C	Gi–inhibiting AC	Presynaptically reduces basal sympathetic outflow at low frequency nerve activity Postsynaptically lowers cAMP	Lowers blood pressure Participation in vasoconstriction after exposure to cold temperatures

As mentioned above for the integrin-ILK signalling for phosphorylation of myosin RLC and MLCP, integrin-dependent signalling involving a tyrosine phosphorylation cascade can also activate the L-type Ca^{2+}-channel. This signalling pathway may translate mechanical forces of the ECM-linked integrins directly to an increase in Ca^{2+} entry, leading to an increase in vascular tone.

Hallmarks of smooth muscle versus myocardial regulation

The following characteristics differentiate smooth muscle regulation from that of the myocardium. (i) Smooth muscle contraction and relaxation are slow processes (in the range of seconds to minutes) compared with heart muscle (in the range of milliseconds). In heart muscle the on–off switch is effected by allosteric conformational changes in the contractile protein complex (myosin-II, actin, troponin and tropomyosin), by fast and reversible binding of Ca^{2+} to cardiac TnC. Regulation in smooth muscle operates exclusively via covalent protein modification by reversible phosphorylation of myosin RLC and/or actin filament components. These processes require the activation of enzyme systems for sequential phosphorylation and dephosphorylation steps. (ii) Vascular smooth muscle tone can be achieved by various agonists via GPCR and Gαq-induced signalling in a Ca^{2+}-independent manner. The degree of vasoconstriction depends primarily on the activity ratio between MLCK and MLCP. The term 'Ca^{2+}-sensitization' (an increase in force without an increase in cytoplasmic Ca^{2+}) is referred to in the context of inhibition of the MLCP. (iii) Smooth muscle tone depends on very slow turnover of the myosin crossbridges with a low degree of phosphorylation and, consequently, low ATP consumption (an energy-saving mechanism). (iv) In large conduit arteries it seems that force maintenance depends predominantly on actin filament regulation. In contrast, in small resistance arteries the α_1-AR-mediated signalling via the Rho–ROK pathway, resulting in Ca^{2+}-sensitization of myosin-II, appears to prevail. (v) Unlike in heart muscle, where β-AR signalling increases total intracellular Ca^{2+} in proportion to the inotropic effect, in vascular smooth muscle β-AR signalling leads to relaxation by inhibiting the activation of myosin-II, independent of the cellular Ca^{2+}. This last point represents the basis for understanding some of the major unwanted side effects of β-AR blocker treatment, such as peripheral vasoconstriction, increased bronchial resistance and the risk

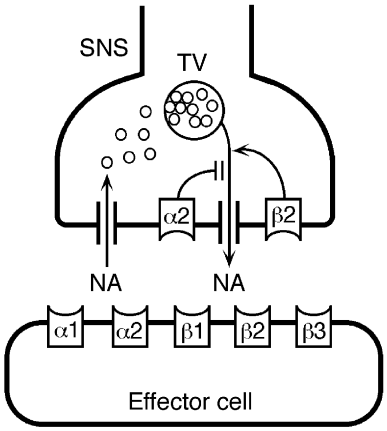

Fig 6 Schematic representation of adrenergic synaptic transmission in the central and peripheral nervous system and at the site of target tissues. The effector cells may comprise cardiomyocytes, smooth muscle cells or endothelial cells. NA=noradrenaline; SNS=sympathetic nervous system; TV=transmitter vesicle. Line with blunted end (=) indicates inhibition.

of hypoglycaemia with diabetes mellitus. On the other hand, the treatment of asthma with β_2-AR agonists for bronchodilatation may entail tachycardia (Table 1).

Interplay between calcium and adrenergic receptor subtype-specific signalling

In general, the type-2 ARs (α_2 and β_2) are found at the prejunctional site in the central and peripheral sympathetic nervous system, where activation of α_2-AR inhibits and activation of β_2-AR enhances noradrenaline release, both being of pharmacological significance (Fig. 6). In most effector cells, such as cardiomyocytes, endothelial and smooth muscle cells, the type-2 ARs are also present postsynaptically together with α_1, β_1 and β_3-ARs (Figs 1 and 6). Stimulation of the α_2-ARs lowers intracellular Ca^{2+} by activation of Gαi-proteins, inhibition of the AC, activation of sarcolemmal inwardly rectifying K^+-channels, and inhibition of the L-type Ca^{2+}-channels, resulting in lowering of the Ca^{2+}-dependent noradrenaline release from the presynaptic transmitter vesicles (Table 1). Presynaptically localized α_{2A}-AR and α_{2C}-AR subtypes are important in lowering sympathetic tone in the central nervous system and in decreasing noradrenaline release in cardiac sympathetic nerve terminals.[24 69 74] Use of the isotope dilution method demonstrated that the sympathetic outflow in chronically failing hearts is greatly enhanced with a cardiac noradrenaline spillover, being increased by as much as 50-fold. On the other hand, the stimulation level of cardiac sympathetic nerves is one of the most powerful predictors of mortality.[19] The α_2-AR agonists clonidine and dexmedetomidine are non-subtype-selective. They are used to treat patients with hypertension, glaucoma (local application), tumour pain or postoperative pain, and to block sympathetic overactivity during opioid withdrawal.[68 80] While postsynaptic α_{2B}-ARs are responsible for the short-term hypertensive effect of α_2-agonists (vasoconstriction but concomitant nitric oxide release), postsynaptic α_{2A}-ARs mediate primarily the sympathetic tone of the central nervous system. However, there are no direct α_2-AR-mediated effects on the myocardium.

The β-ARs are the key regulators of heart rate, systolic and diastolic function, and myocardial metabolism. Extracellular adrenergic stimuli (catecholamines) intermingle with intracellular Ca^{2+} signalling. Not only is Ca^{2+} a key intracellular messenger for many signalling components, but its own spatiotemporal signalling transients are decisively influenced by the G-protein coupled β-ARs. An integrated view of the complex interplay between adrenergic and Ca^{2+} signalling is schematized in Figs 1–3 and 5. Acute changes in myocardial function are almost exclusively governed by the β-AR signalling pathways. A functionally relevant contribution of α_1-ARs appears unlikely in humans under normal conditions. However, in heart failure, β-ARs are desensitized and downregulated ($\beta_1 > \beta_2$), whereas the total amount of α_1-AR subtypes remains constant or may even be upregulated. Under these conditions, the α_1-AR-mediated inotropy may prove important.[11 54 74 92] In the rat myocardium, α_{1A}- and α_{1B}-ARs display significant positive effects on inotropy, heart rate and growth (Table 1). Positive inotropy by these two α_1-AR subtypes was also shown for mouse, hamster, guinea pig, rabbit and dog. It was recently shown that the positive inotropic response of rat papillary muscle to stimulation of the α_{1A}-AR subtype is primarily due to myosin RLC phosphorylation by MLCK.[1] This RLC phosphorylation depends neither on cAMP nor on PKC activation, and the cytoplasmic Ca^{2+} is only moderately increased. RLC phosphorylation sensitizes the contractile apparatus to Ca^{2+}, i.e. enhanced contraction in the presence of unchanged cytoplasmic Ca^{2+} concentration. Similar observations were made with endothelin stimulation. Taking these observations together, it is hypothesized that in myocytes the positive inotropy elicited by α_1-ARs, in general, depends on the signalling cascade Gαq–PLC–IP3–Ca^{2+}–CaM and subsequent phosphorylation of the myosin RLC by MLCK. This is in contrast to the smooth muscle, where the α_1-mediated increase of contractility is mainly achieved by inhibition of the MLCP system by the Rho–ROK pathway and by activation of specific PKC isoforms (Fig. 5). All three α_1-AR subtypes are found in smooth muscle cells and mediate vasoconstriction (Table 1).[2 11 24 47 69]

An interesting aspect concerns the signalling differences between β_1- and β_2-AR. Although both β_1-ARs and β_2-ARs increase contractile amplitude and hasten relaxation in ventricular myocytes, several striking differences with respect to signal transduction downstream from the β-ARs have been revealed. While β_1-AR signalling strictly adheres to the canonical cAMP–PKA pathway, the β_2-AR adopts a much more flexible approach by being able to signal via Gαs, Gαi, Gαq and/or Gβγ depending on spatiotemporal

relations in the receptor microenvironment (Fig. 1). It was demonstrated that apoptotic myocyte cell death is dissociated from β_2-AR and selectively mediated by β_1-AR in isolated ventricular myocytes and in intact hearts *in vivo*.[96 98 99] The possible activation of different G-proteins by β_2-ARs may result in distinct downstream signalling pathways. The chronic catecholamine-dependent positive inotropy of β_2-AR is supported by an increased Ca^{2+} influx via the L-type Ca^{2+}-channel without cardiotoxic consequences. This is achieved by locally restricted signalling to the Ca^{2+}-channel located close by in the membrane, and bypasses the β_1-typical effects on PLN and the contractile proteins. On the other hand, coupling to $G\alpha i/G\beta\gamma$ activates several cytoprotective mechanisms (counteracting in part the $G\alpha s$-mediated routes), such as activation of ATP-dependent K^+-channels in the sarcolemma and in the inner mitochondrial membrane, mediated by different PKC isoforms, and activation of the protein kinase B (PKB, also called Akt). In the latter case, signalling proceeds via the PI3K and PI3K-dependent kinase-1 (PDK1) pathway (Fig. 1). PKB activation leads to increased expression of the antiapoptotic protein Bcl-2 and inhibition of proapoptotic factors, such as caspase-9 and Fas-ligand expression.

Activation of the endothelial nitric oxide synthase (eNOS), which is constitutively expressed in cardiomyocytes, greatly depends on β_3-AR signalling and leads to activation of the cGMP-dependent PKG.[2 47 90] NO may also reach the myocytes from extracellular sources, such as the endothelial cells. Thus, NO operates in both autocrine and paracrine fashions. The NO–GC–cGMP–PKG pathway also exerts a negative inotropic effect in the myocytes by reducing Ca^{2+} influx through L-type Ca^{2+}-channels. This negative inotropic effect of the β_3-AR acts under normal conditions as a negative counter-regulation. In contrast, in the failing heart, where β_3-ARs are upregulated with concomitant downregulation of β_1-ARs, this negative inotropic effect may aggravate the deterioration of cardiac function.

Conclusions

Insight into the complex sympathetic regulatory mechanisms may not only allow better therapeutic approaches in perioperative medicine, but may also help explain the clinically observed unwanted or unexpected side-effects that may occur when using pharmacological modulators of AR activity. Some of the relevant new properties that we have discussed are adrenergic receptor signalling via different types of G-proteins, homologous and heterologous desensitization of ARs, the homo- and heterodimerization of AR-subtypes, and the downstream signalling network connected to the different adrenergically modulated cellular functions. Adrenergic signalling relies in principal on two intracellular signalling modes: (i) reversible binding of Ca^{2+} ions to specific protein sites; and (ii) several different protein phosphorylation cascades. These two signalling modes follow distinct pathways, which are intimately interlinked. On the one hand, the signalling crosstalk entails considerable redundancy, securing cardiac function under abruptly changing or even adverse conditions. On the other hand, it reveals a fine-tuning of regulation that is well suited for differentiated pharmacological interventions. What have not been dealt with in this review are the human polymorphisms in adrenergic receptors and in their downstream signalling components that have recently come to light; these may affect the established adrenergic signalling in some cases. In conclusion, a detailed understanding of the basic adrenergic functions in cardiac and vascular tissues is indispensable for the development of new perioperative therapeutic strategies and will foster the testing of novel hypotheses in randomized clinical trials.

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